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(54) Title: DIAGNOSTIC PROBE FOR DETECTING HUMAN STOMACH CANCER

(57) Abstract

A diagnostic probe for detecting human stomach cancer is described.

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DIAGNOSTIC PROBE FOR DETECTING HUMAN STOMACH CANCER

A number of oncogene and proto-oncogene probes have been reported to be useful in diagnostic procedures for detecting certain forms of cancer and for following disease prognosis. However, a specific probe for the diagnosis of human stomach cancer has not heretofore been known or described.

It is, therefore, an object of the present invention to provide specific probes for detecting human stomach cancer. Such a unique probe came into being by the unexpected finding that the met proto-oncogene was activated in certain human cancer cell lines tested. The characteristics of the met gene can be found described in Park et al, 1986, Cell, 45:895-904; Park et al, 1987, PNAS, 84:6379-6383 and Gonzatti-Haces et al, 1988, PNAS, 85:21-25.

Materials & Methods

Various methodologies used for testing the activation of met gene are now described.

Southern Blotting

Genomic DNA from the HOS line and several gastric carcinoma cell lines were digested with EcoRI, fractionated on a 1% agarose gel, and transferred to nitrocellulose. The blot was baked for 2 hours. The blot was prehybridized for 4 hours at 42°C. The prehybridization and hybridization was in 50% formamide, 5x Denhardts, 200 μg/ml salmon sperm DNA, 5x SSPE, and 0.1% SDS. The blot was washed with 2x SSC, 0.1% SDS for 20 minutes at RT, then with 3 washes of 0.2 x SSC, 0.1% SDS at 68°C.

Northern Blotting

Total RNA was fractionated on a 1.1% formaldehyde-agarose gel and transferred to nitrocellulose. The nitrocellulose was baked for 2 hours, then washed 20 minutes in 5x SCC. The blot was prehybridized for 4 hours at 42°C. Prehybridization and hybridization were in 50% formamide, 0.1% SDS, 6x SSPE, 1x Denhardts, and 200 μg/ml sonicated salmon sperm DNA. The blot was washed 2 times for 10 minutes in 2x SCC, 0.1% SDS at room temperature (about 22°-24°C), then 3 times for 20 minutes each in 0.2x SSC, 0.1% SDS at 55°C.

^{35}S Metabolic Labeling and Immunoprecipitation

Cells were preincubated 15 minutes in DMEM lacking methionine and cysteine supplemented with 10% calf serum, then labeled for 30 minutes in the above media supplemented with 2.5 mCi/ml ^{35}S-methionine and cysteine. Cells were lysed and met was then immunoprecipitated with a monoclonal directed against the kinase domain. Immunoprecipitates were fractionated on a 3-17% gradient gel. Proteins were detected by fluorography.

^{32}P Metabolic Labeling

Cells were preincubated for 15 minutes with DMEM lacking phosphate, then labeled for 2 hours in the above media supplemented with 0.5 mCi/ml ^{32}P-orthophosphate.

Monoclonal Antibody to the met Oncogene Product

A deposit of the hybridoma producing monoclonal antibody having specificity to the met oncogene product has been made at the ATCC, Rockville, MD., on December 14, 1989 under accession number HB 10309. The deposit shall be viably maintained, replacing if it becomes

non-viable during the life of the patent, for a period of 30 years from the date of the deposit, or for 5 years from the last date of request for a sample of the deposit, whichever is longer, and upon issuance of the patent made available to the public without restriction in accordance with the provision of the law. The Commissioner of Patents and Trademarks, upon request, shall have access to the deposit.

RESULTS

Figure 1 shows the gene copy number in gastric carcinoma cell lines as determined by Southern blot. A fragment representing most of the met cDNA was used to probe genomic DNA which had been digested with EcoRI. Several hybridizing bands are detected. The MKN-45 and Okajima cell lines show significant amplification of the met gene. No rearrangement is detected with this probe. To further confirm these results, RNA levels were examined by Northern blot. Total RNA from several cell lines was probed with a fragment containing the met extracellular domain. A 9 kb met RNA is detected in all cell lines. As shown in Fig. 2, the met RNA is greatly overexpressed in the MKN-45 and Okajima cell lines.

Then the expression and half-life of the met protein in some of these lines was examined by pulse chase. Cells were labeled with ^{35}S for thirty minutes and then chased with cold media for 1/2, 2 or 4 hours. As shown in Fig. 3, very high levels of the met protein are detected in the MKN-45 line. The protein appears to be processed normally. The MKN-74 and KATOIII lines shown normal protein levels. The Okajima line shows the greatest amount of protein after a 30 minute

labeling, indicating a high rate of synthesis. While the met protein in this line is processed to the 140 form, the 140 is almost all gone after a four-hour chase, indicating a very short half-life.

It was then examined whether the proteins might be phosphorylated on tyrosine. Cells were labeled for 2 hours with ^{32}P orthophosphate and protein was precipitated with an antibody directed against phosphotyrosine. As seen in Fig. 4, both the MKN-45 and Okajima lines show phosphotyrosine present on the p140 form. No phosphotyrosine was detected on the p140 in the other cell lines, even with a long overexposure.

Table 1 summarizes the data presented in Figs. 1-4. Of the 7 lines examined, two (MKN-45 and Okajima) showed amplification and phosphorylation on tyrosine on p140. The Okajima line was unique in displaying a very short half-life for p140. When these lines were examined for their ability to grow in serum-free media, only the Okajima line was found competent to grow. It is significant to note that met was found amplified only in poorly differentiated adenocarcinomas. The other lines examined for which a classification is known included two well differentiated adenocarcinomas and signet ring carcinomas.

The results indicate that the met gene is amplified and overexpressed only in poorly differentiated gastric carcinoma cell lines tested. The rapid turnover seen in one line may indicate that an autocrine loop is involved in the genesis of the tumor. Since the met amplification was seen only in poorly differentiated adenocarcinomas, clearly a met probe may be of great value in characterizing the clinical stage to which a

tumor has progressed. The availability of the monoclonal antibodies to the met gene product now makes it possible not only to isolate and purify, but also to detect the presence of the met gene products in a biological sample by standard immunological techniques including in situ immunofluorescence or other standard techniques. Accordingly, a diagnostic kit for the detection of met gene product, comprises a container containing antibodies to the met gene product.

Of course, given the nucleotide and amino acid sequences, a nucleic acid or polypeptide probe for detecting the met gene or the met gene product is easily made by conventional methodologies well known to one of ordinary skill in the art. Nucleic acid probes useful for this purpose are described in Park et al, supra, while probes are also described in Gonzatti-Haces et al, supra.

It is noted that unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials have been described. Unless mentioned otherwise, the techniques employed or contemplated herein are standard methodologies well known to one of ordinary skill in the art. The materials, methods and examples are illustrative only and not limiting.

It is understood that the examples and embodiments described herein are for illustrative purposes only and

that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

TABLE 1

	Cell LINE	MET AMPLIFIED	p140 SHORT HALF LIFE	p140 P-TYR	SERUM FREE GROWTH	CLASSIFICATION
	HOS	-	-	-	-	OSTEOSARCOMA
GASTRIC CARCINOMAS	MKN-7	-	nd	nd	-	WELL DIFFERENTIATED ADENOCARCINOMA
	MKN-45	+	-	+	-	POORLY DIFFERENTIATED ADENOCARCINOMA
	MKN-74	-	-	-	-	WELL DIFFERENTIATED ADENOCARCINOMA
	KATO III	-	-	-	-	SIGNET RING CARCINOMA
	OKAJIMA	+	+	+	+	POORLY DIFFERENTIATED ADENOCARCINOMA
	MGC 80-3	-	nd	nd	nd	
	BGC 82-3	-	nd	nd	nd	

nd= not done

WHAT IS CLAIMED IS:

1. A method for detecting clinical stages of human stomach cancer, comprising the step of determining the over expression of met gene in a human stomach tumor specimen by a suitable probe, an overexpression of met gene in said specimen being indicative of the cancerous stage.
2. A DNA fragment which binds specifically with the met gene.
3. An antibody which binds specifically with the met gene product.
4. A diagnostic kit for detecting the presence of met gene product in a biological sample, comprising a container containing the antibody of claim 3.
5. A method for detecting the presence of met gene product in a biological sample, comprising reacting a biological sample in which the presence of met gene product is to be determined, with the antibody of claim 3, a positive immunological reaction being indicative of the presence of met gene product in said sample.
6. A method for isolating purified met gene product, comprising the step of adsorbing met gene product utilizing the antibody of claim 3 and then recovering the adsorbed met gene product therefrom in a purified form.

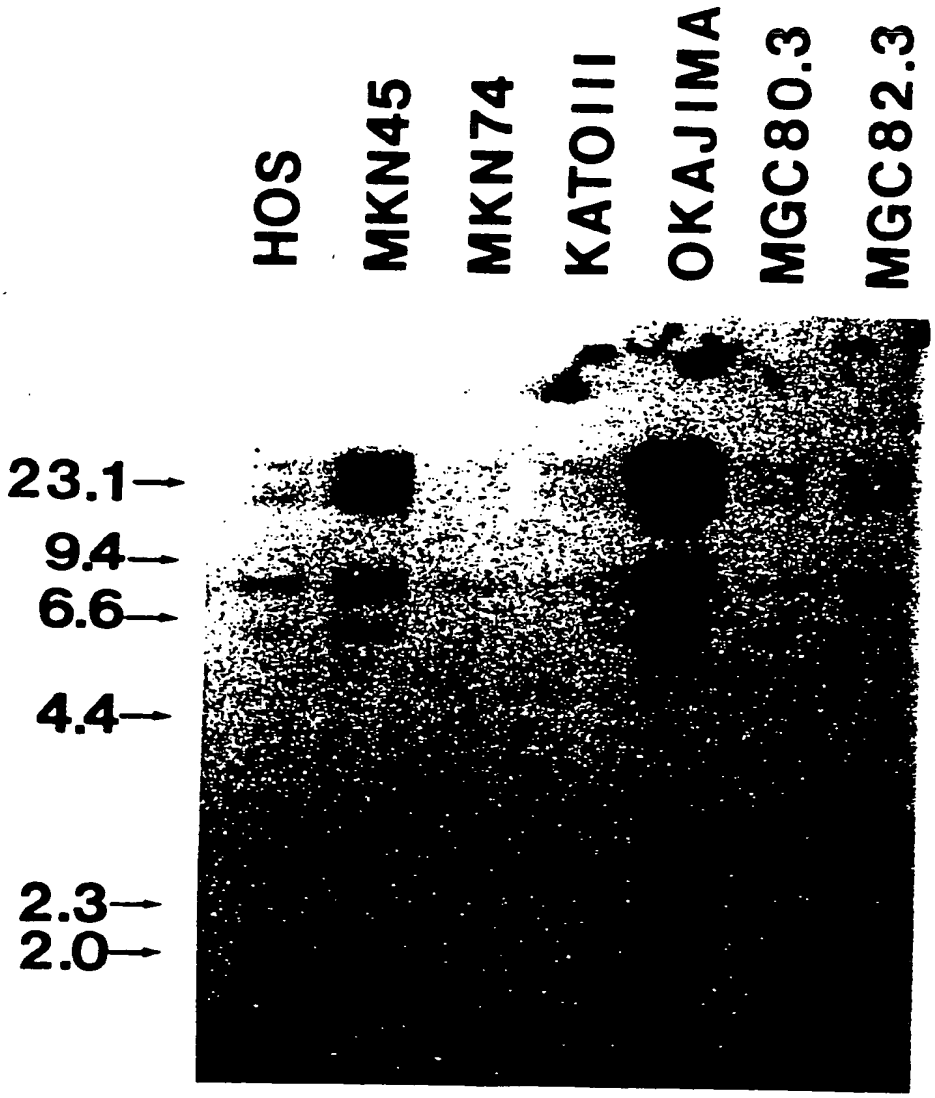


FIG. 1

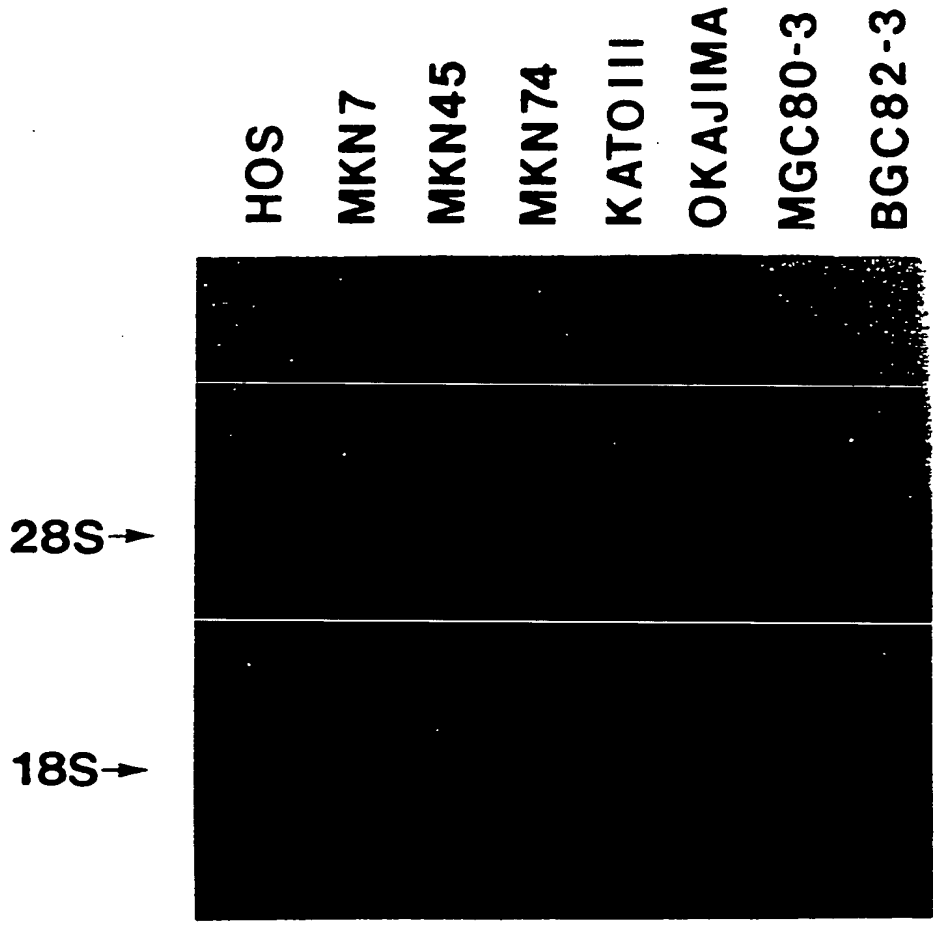
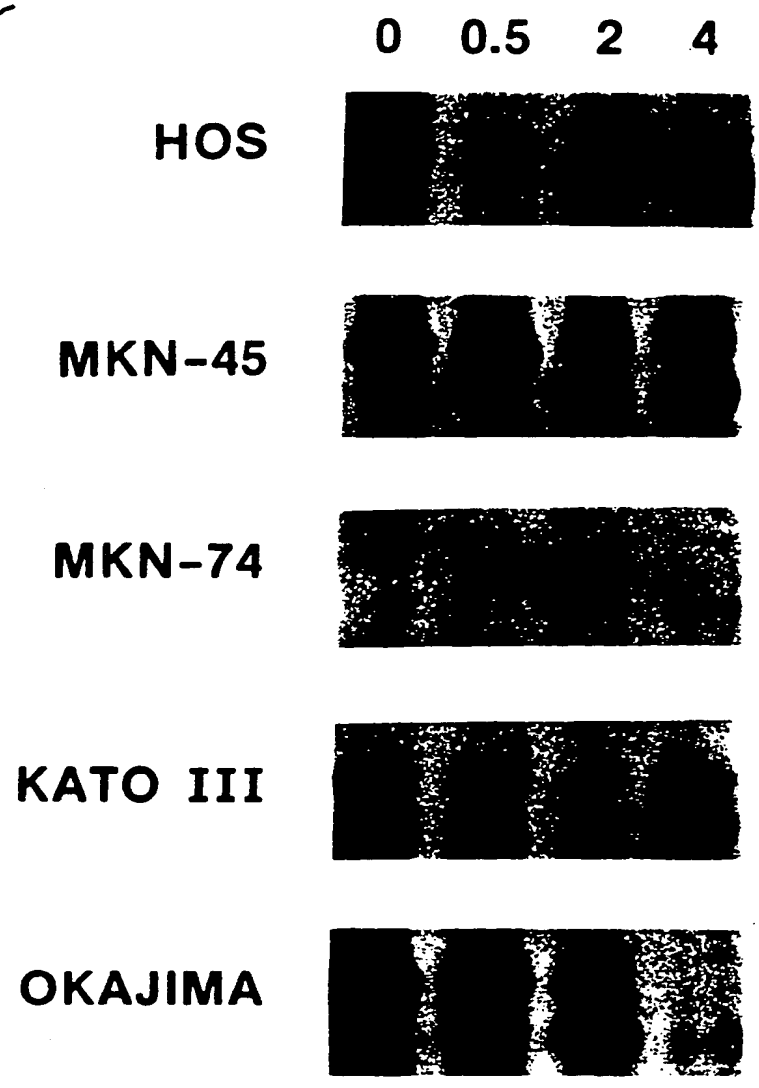


FIG. 2

FIG. 3



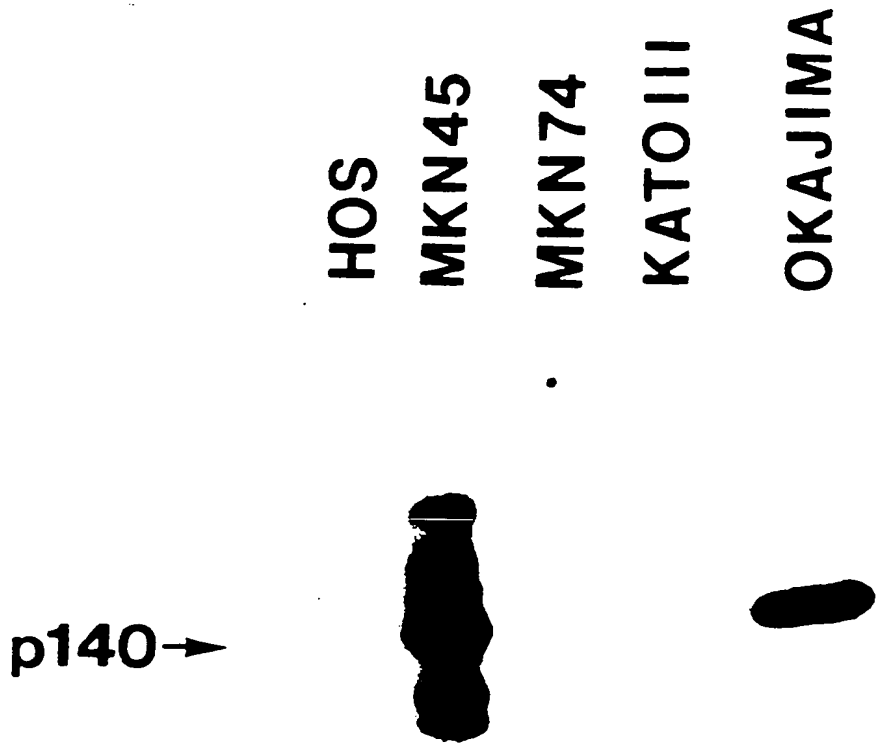


FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT US90 07313

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/68; C07H 15/12; C12N 15/00; A61K 39/00

U.S. CL.: 435/6; 536/27; 935/77, 78; 424/85.8

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	435/6; 536/27; 935/77, 78; 424/85.8

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

APS, STN

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y	THE EMBO JOURNAL, Vol. 5, No. 10 issued 1986, **COOPER ET AL.**, "Amplification and overexpression of the met gene in spontaneously transformed NIH3T3 mouse fibroblasts", pages 2623-2628, see the entire document.	2 1
X	FEBS Letters, Vol. 209, No. 2, issued December 1986, **TEMPEST ET AL.**, "The activated human met gene encodes a protein tyrosine kinase", pages 357-361, see the entire document.	3-6
X Y	Nucleic Acids Research, Vol. 16, No. 17, issued 1988, **SWEET ET AL.**, "An EcoRI polymorphism for pMet G inbred mice", page 8745, See the entire document.	2 1

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

14 March 1991

Date of Mailing of this International Search Report

16 APR 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Mindy B. Fleisher (vsh)

Form PCT/ISA/210 (second sheet) (rev. 11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1 and 2 drawn to a method and nucleic acid probe, classified in Class 435, subclass 6.

II. Claims 3-6 drawn to an antibody and method of use, classified in Class 424, subclass 85.8.

1 ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2 ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3 ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4 ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.